(38) GVHD

In Vivo Neutralization of TNF-α Promotes Humoral Autoimmunity by Preventing the Induction of CTL[1]

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Neutralization of TNF-α in humans with rheumatoid arthritis or Crohn's disease has been associated with the development of humoral autoimmunity. To determine the effect of TNF-α neutralization on cell-mediated and humoral-mediated responses, we administered anti-TNF-α mAb to mice undergoing acute graft-vs-host disease (GVHD) using the parent-into-F_1 model. In vivo neutralization of TNF-α blocked the lymphocytopenic features characteristic of acute GVHD and induced a lupus-like chronic GVHD phenotype (lymphoproliferation and autoantibody production). These effects resulted from complete inhibition of detectable antihost CTL activity and required the presence of anti-TNF-α mAb for the first 4 days after parental cell transfer, indicating that TNF-α plays a critical role in the induction of CTL. Moreover, an in vivo blockade of TNF-α preferentially inhibited the production of IFN-γ and blocked IFN-γ-dependent up-regulation of Fas; however, cytokines such as IL-10, IL-6, or IL-4 were not inhibited. These results suggest that a therapeutic TNF-α blockade may promote humoral autoimmunity by selectively inhibiting the induction of a CTL response that would normally suppress autoreactive B cells. *The Journal of Immunology*, 2001, 167: 6821–6826.

Tumor necrosis factor-α is a pleiotropic cytokine that plays a major role in inflammatory responses (1). Recently, agents that neutralize TNF-α have been found to be beneficial in patients with rheumatoid arthritis and Crohn's disease (2, 3). A surprising side effect of such treatment in some patients is the development of humoral autoimmunity characterized by production of antinuclear and anti-dsDNA Ab production (4–9). Less commonly, treated individuals have developed clinical features of systemic lupus erythematosus (SLE)[3] (4). Although these observations are consistent with previous reports of a link between SLE and reduced TNF-α production (10), the mechanism responsible for development of humoral autoimmunity in patients treated with TNF antagonists is not established.

Suggestions that the inability to suppress or eliminate B cell hyperactivity because of an ineffective CTL response may contribute to SLE pathogenesis (11) and observations that membrane TNF-α expression by CTL can contribute to target cell lysis (12) raise the possibility that TNF-α antagonists may promote humoral autoimmunity by inhibiting CTL responses. To test this hypothesis, we examined TNF-α regulation of graft-vs-host disease (GVHD) in the parent-into-F_1 murine model. This system, in which homozygous parental T cells are inoculated into unirradiated heterozygous mice, can lead to the development of either a cell-mediated (acute GVHD) or an Ab-mediated (chronic GVHD) antihost response, depending upon the parental mouse strain used. For example, inoculation of (C57BL/6 × DBA/2)F_1 mice with C57BL/6 parental T cells induces acute GVHD while inoculation of the same F_1 hosts with DBA/2 parental T cells induces chronic lupus-like GVHD (13). Both acute and chronic GVHD are characterized initially by B cell hyperactivity and autoantibody production. In acute GVHD, however, donor cells develop within 7 days into antihost CTL that eliminate most host B cells, including autoreactive B cells, during the subsequent 5–7 days (14). In chronic GVHD, in contrast, antihost CTL fail to develop and continued autoantibody production results in a lupus-like immune complex glomerulonephritis. Previous studies demonstrating that selective in vivo inhibition of $CD8^+$ T cell-CTL development prevents acute GVHD and leads to the development of chronic lupus-like GVHD support the view that $CD8^+$ CTL control autoreactive B cell hyperactivity and that the absence of such CTL plays a permissive role in humoral autoimmunity development (15). We now demonstrate that TNF-α is required to suppress humoral autoimmunity in GVHD and does so by inducing CTL development rather than by contributing to CTL effector function.

Materials and Methods

Mice

C57BL/6J (B6) and C57BL/6 × DBA/2 (BDF_1) male mice, 6–8-wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Induction of GVHD

Single-cell suspensions were prepared in HBSS from the spleens of normal B6 parental donors. Cell suspensions were filtered through sterile nylon mesh, washed, and diluted to a concentration of 10^8 viable (trypan blue excluding) cells/ml. Acute GVHD was induced by injecting 50×10^6 B6 splenocytes i.v. into the tail veins of normal unirradiated BDF_1 recipients (16). Chronic GVHD was induced using 50×10^6 CD8-depleted B6 splenocytes

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; GVHD, graft-vs-host disease; CCCA, Cincinnati cytokine capture assay; FasL, Fas ligand.

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(18). Unless otherwise stated, mice received 2 mg of the rat IgG1 anti-TNF-α mAb, MP6-XT22 (17), or control rat IgG1 mAb (GL113) i.v. beginning at the time of parental cell transfer (day 0) and repeated every 3–4 days thereafter for a total of four doses. Positive and negative controls were, respectively, BDF_1 mice injected with B6 spleen cells in the absence of mAbs and uninjected age- and sex-matched BDF_1 mice.

Detection of antihost CTL activity ex vivo

Effector CTL activity of freshly harvested splenocytes was tested in a 4-h ^{51}Cr release assay, as described in Ref. 18, without an in vitro sensitization period. Splenocytes from control and GVHD mice were tested for their ability to lyse Fas-dull P815 cell line ($H\text{-}2^d$, MHC class I positive, class II negative) targets. Using serial dilutions, effectors were tested in triplicate at four E:T ratios, beginning at 100:1 (1.5×10^6 effectors and 0.015×10^6 targets/well). The percentage of lysis was calculated according to the formula: (cpm sample − cpm spontaneous)/(cpm maximum − cpm spontaneous) × 100%. Results are shown as the mean percent lysis ± SEM at a given E:T ratio for each treatment group.

Cincinnati cytokine capture assay (CCCA)

A recently described assay, the CCCA (19) was used to quantitate in vivo production of IFN-γ, TNF-α, IL-2, IL-4, and IL-6 in mice undergoing GVHD. The CCCA increases the sensitivity of detection of each of the cytokines measured by a factor of 100–1000. Briefly, mice are injected i.v. with 10 μg of a biotin-labeled neutralizing mAb to IL-2, IL-4, IL-6, TNF-α, or IFN-γ, which binds some, but not all, of the respective cytokine shortly after it is secreted. The biotin-mAb–cytokine complexes formed have a much longer in vivo half-life than uncomplexed cytokines and accumulate in serum. Mice are bled 1 day after biotin-mAb injection and concentrations of biotin-mAb–cytokine complexes are measured by ELISA using microtiter plate wells coated with mAbs to an epitope on the appropriate cytokine that is not blocked by the injected biotin-labeled mAb to the same cytokine. Biotin-labeled mAb–cytokine complexes in serum samples or standards (prepared by mixing recombinant cytokines, purchased from BD PharMingen (San Diego, CA), with the appropriate biotin-anti-cytokine mAbs at a 1:100 weight ratio) are detected with streptavidin-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by a substrate solution (SuperSignal ELISA Femto Maximum Sensitivity Substrate; Pierce, Rockford, IL) that generates a luminescent compound when cleaved by HRP. Plates are read immediately after addition of substrate with a Fluoroskan Ascent FL luminometer (Labsystems, Helsinki, Finland).

The CCCA does not interfere with ongoing immune responses because only a relatively small percentage of secreted cytokine is bound by the injected biotin-mAb and because biotin-mAb–cytokine complexes contain only 1 molecule of IgG mAb and thus do not fix, complement, or bind to FcγR more avidly than endogenous serum IgG. The following pairs of anti-cytokine mAbs were used, all of which were obtained from Dr. D. Sehy at BD PharMingen: for IL-2, inject biotin-JES6-5H4, then coat wells with JES6-1A12; for IL-4, inject biotin-BVD4-1D11, then coat wells with BVD6-24G2.3; for IL-6, inject biotin-MP5-32C11, then coat wells with MP5-20F3; for IFN-γ, inject biotin-AN-18, then coat wells with R46A2; and for TNF-α, inject biotin-TN3, then coat plates with the IgG fraction of rabbit anti-TNF-α polyclonal antiserum. References for all of the mAbs used for this assay are given in the BD PharMingen catalog; detailed protocols for each cytokine are available on *The Journal of Immunology* Web site and will be published (F. Finkelman, S. Morris, T. Orekhtova, and D. Sehy, manuscript in preparation).

RT-PCR

Cytokines were also measured by semi-quantitative RT-PCR as previously described (20). Briefly, RNase-free plastic and water were used throughout the assay and tissues were homogenized in RNA-STAT-60 (Tel-Test, Friendswood, TX) at 50 mg tissue/ml or 1 ml/10^7 cells. RNA samples were reverse transcribed with reverse transcriptase (Life Technologies, Grand Island, NY); Fas ligand (FasL) or IFN-γ specific primers were used for amplification as described in Ref. 20. For each gene product, the optimum number of cycles (that number of cycles that would achieve a detectable concentration that was well below saturating conditions) was determined experimentally. To verify that equal amounts of RNA were added in each RT-PCR within an experiment, primers for the housekeeping gene *hypoxanthine phosphoribosyltransferase* were used in each experiment. Gene expression was quantitated by densitometry for individual mice, normalized to each individual *hypoxanthine phosphoribosyltransferase* value, and group means calculated.

Flow cytometry analysis and engraftment studies

Spleen cells were prepared as described in Ref. 16. Following incubation with the anti-murine FcγRII/RIII mAb, 2.4G2, (21) for 10 min, cells were stained with saturating concentrations of FITC-, biotin-, or PE-conjugated mAb against CD4, CD8, B220, Fas, or $H\text{-}2K^d$ purchased from BD Biosciences (Mountain View, CA.) or BD PharMingen. Two-color flow cytometry was performed using a FACScan (BD Biosciences), lymphocytes were gated by forward and side scatter, and fluorescence data were collected on 10,000 cells. Donor T cells were defined as $CD4^+$ or $CD8^+$ and stained negatively for MHC class I expressed by the recipient, but not the donor, cells. Host B cells were identified as B220-positive host I-A-positive cells. Monocyte populations were excluded on the basis of forward and side scatter.

Serological studies

Mice were bled at the times indicated and sera were tested by ELISA for the presence of IgG antibodies to ssDNA as described in Ref. 16. Briefly, microtiter plates were coated with heat-denatured salmon sperm DNA, blocked with 2% BSA-PBS and incubated with 2-fold serial dilutions of experimental mouse sera beginning at a dilution of 1/40. The plates were then incubated with alkaline phosphatase-labeled anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) and OD quantitated at 405 nm. For each experiment, pooled MRL/*lpr* sera were tested in parallel and a standard curve constructed for conversion of experimental sera OD values to units. An arbitrary value of 1000 U was assigned to MRL/*lpr* sera at a dilution of 1/2000.

Statistical Analysis

Data were examined for normality and equal variance (Kolmogorov-Smirnov test). If satisfactory, groups were compared by two-tailed Student's *t* test, if not they were compared by the Mann-Whitney rank sum test.

Results

Neutralization of TNF-α in vivo inhibits the development of acute GVHD and enhances anti-DNA Ab production in a dose-dependent fashion

To determine the role of TNF-α in the development of acute GVHD, BDF_1 mice were inoculated with B6 spleen cells and received either no additional treatment or 0.125–2 mg of anti-TNF-α mAb twice a week, beginning on the day of parental cell transfer (Table I). Treatment with the highest dose of anti-TNF-α mAb (2 mg) not only blocked the reduction in total splenocytes and host B cells typically seen in acute GVHD, but also resulted in lymphoproliferation, as evidenced by an ~40–50% increase in both total spleen cells and host B cells compared with normal untreated F_1 mice ($p < 0.005$ for both). Treatment with 0.125 mg of anti-TNF-α mAb did not significantly alter GVHD-associated splenic lymphopenia or B cell elimination. Intermediate doses (0.5 mg) of anti-TNF-α mAb resulted in a trend toward less severe acute GVHD; however differences were not statistically significant when compared with untreated or low-dose mAb-treated acute GVHD mice.

The lymphoproliferation and B cell expansion observed in mice injected with the highest dose of anti-TNF-α mAb was associated with a significant increase in serum anti-ssDNA Ab levels compared with either untreated acute GVHD mice (2.5-fold) or with normal F_1 mice (8-fold; $p < 0.05$ for both; Table I). Mice treated for 10 days with high-dose anti-TNF-α mAb still exhibited increased spleen cell numbers and anti-DNA Ab titer 1 month later (data not shown). Taken together, these data indicate that stringent neutralization of TNF-α induces features of chronic GVHD in mice that would otherwise develop acute GVHD and suggest that less complete TNF-α neutralization can block features of acute GVHD without inducing lupus-like GVHD.

Table I. *Anti-TNF-α mAb blocks the lymphocytopenic features of acute GVHD in a dose-dependent manner*[a]

Group[a]	Splenic Yield	Donor		Host	
		CD4+	CD8+	B cells	Anti-DNA[b]
Normal F1	91.8 (7.2)	ND[a,c]	ND	48.9 (3.9)	6.4 (0.9)
Acute GVHD	57.7 (10.2)	6.2 (0.4)	10.5 (0.8)	15.8 (5.4)	20.4 (8.8)
Acute GVHD + anti-TNF (2 mg)	133.0 (3.5)[d]	2.4 (0.5)	2.1 (0.6)	65.7 (5.1)[d]	55.5 (10.2)[d]
Acute GVHD + anti-TNF (0.5 mg)	72.3 (8.8)	5.7 (1.3)	7.4 (1.0)	27.3 (5.3)	18.2 (6.0)
Acute GVHD + anti-TNF (0.125 mg)	47.8 (10.3)	4.5 (0.8)	3.8 (0.4)	13.0 (2.5)	9.7 (1.1)

[a] Acute GVHD was induced as described in *Materials and Methods* and mice received anti-TNF-α mAb (MP6-XT-22) i.v. at the indicated dose on days 0, 3, 7, and 10 after parental cell transfer. Splenocytes were analyzed by flow cytometry 14 days after parental cell transfer. Values for total splenocytes and donor or host lymphocyte subsets are shown as group mean (±SE) × 10^{-6}; $n = 4$ mice/group for all groups except untreated acute GVHD ($n = 3$). Similar results were observed in two additional independent experiments.

[b] Anti-ssDNA Ab levels are expressed as units per milliliter (see *Materials and Methods*).

[c] Not detectable above background.

[d] $p < 0.05$ compared to untreated acute GVHD.

Neutralization of TNF-α in vivo blocks antihost CTL activity in acute GVHD

Lymphopenia in acute GVHD is mediated, in large part, by elimination of host cells by host-specific donor CTLs (16, 22). Because selective inhibition of donor antihost CTL activity can permit the development of chronic GVHD (14, 23), the above results were consistent with the possibility that neutralization of TNF-α promotes chronic GVHD by inhibiting donor antihost CTL development. To test this idea, mice received 2 mg of anti-TNF-α or control mAb twice a week and antihost CTL responses were assessed at the time of maximal CTL activity, 10 days after parental cell transfer (14). As shown in Fig. 1, in vivo anti-TNF-α mAb treatment completely inhibited antihost CTL activity, as compared with mice that received parental cells but either no mAb or control mAb. In contrast to its ability to inhibit the induction of CTL activity when administered in vivo before the development of acute GVHD, anti-TNF-α mAb did not block the antihost CTL effector function as evidenced by an inability to: 1) block anti-DBA cytolytic activity when added to IL-2-stimulated cultures of spleen cells from day 10 acute GVHD mice (data not shown) or 2) block antihost CTL effector function when added during the 4-h assay phase (data not shown).

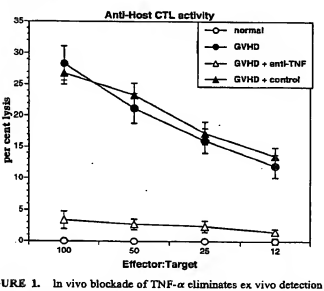

FIGURE 1. In vivo blockade of TNF-α eliminates ex vivo detection of antihost CTL activity in acute GVHD mice. B6>BDF_1 mice were treated with 2 mg of anti-TNF-α (MP6-XT22) or control mAb (GL113) i.v. at days 0, 4, and 7 after parental cell transfer. At 10 days, mice were sacrificed and splenocytes tested for ex vivo killing of H-2^d targets as described in *Materials and Methods*. No significant killing of H-2^b targets was observed. Results are shown as the group mean ± SE at a given E:T ratio ($n = 5$ mice/group). Similar results were seen in two additional experiments.

TNF-α is critical in the inductive phase of antihost CTL generation

Although TNF-α has been shown to contribute to CTL effector function (12), the foregoing data suggest that TNF-α is required in vivo to generate CTLs. Moreover, we have observed that a single dose of anti-TNF-α mAb given the same day as parental cell transfer can block the lymphocytopenic features of acute GVHD and promote autoantibody production almost as effectively as twice weekly mAb administration. Specifically, host B cell numbers in acute GVHD mice that received a single dose of anti-TNF-α mAb did not differ significantly from host B cell numbers in acute GVHD mice that received multiple doses of anti-TNF-mAb (data not shown). These results suggest that TNF-α is important early in CTL development. To test this idea, additional experiments were performed in which the administration of anti-TNF-α mAb was variably delayed after parental cell transfer to determine the critical time period for TNF-α in the generation of donor antihost CTL. Using depletion of host B cells as a measure of in vivo antihost CTL generation and acute GVHD, a single dose of anti-TNF-α mAb was able to significantly inhibit acute GVHD if administered at either day 0, 2, or 4 after parental cell transfer, but failed to block acute GVHD if administered 7 or more days after parental cell transfer (Fig. 2). Because mature donor antihost CTLs are first detected in this system 10 days after donor cell transfer and eliminate host lymphocytes from days 10 to14 (14), these data indicate that in vivo TNF-α blockade prevents acute GVHD by inhibiting CTL induction but not CTL effector function.

TNF is produced early in acute GVHD

Increased serum levels of TNF-α have been reported in acute GVHD mice as early as day 10 after parental cell transfer but were not seen at day 8 (24). Although the foregoing data strongly argue that TNF-α is present in the first few days of acute GVHD, we have been unable to detect increased serum TNF-α at days 3, 5, or 7 by standard ELISA (data not shown). Recent modifications of the CCCA significantly increase the sensitivity of this assay (19). Using this approach, we have determined that as early as day 6, both

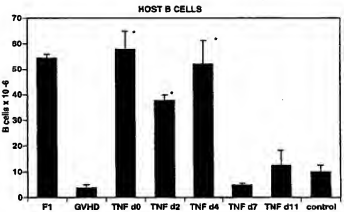

FIGURE 2. A single dose of anti-TNF-α during the first 4 days after parental cell transfer prevents induction of antihost CTL and subsequent elimination of host B cells. Acute GVHD was induced and mice received either no mAb or a single dose of anti-TNF mAb or control mAb. Shown from *left* to *right* are the number of host B cells (mean ± SE × 10^{-6}) at 14 days after parental cell transfer for normal F_1 mice; untreated acute GVHD mice; acute GVHD mice receiving a single dose of anti-TNF-α mAb at days 0, 2, 4, 7, or 11 after parental cell transfer; or acute GVHD mice receiving a single dose of control mAb at day 0 (n = 4–5 mice/group). *, $p < 0.001$ compared with untreated acute GVHD.

acute and chronic GVHD mice exhibit significant increases in several major cytokines. In particular, acute GVHD mice exhibit significantly elevated serum TNF-α levels which are ≥3-fold greater than control mice and ≥2-fold greater than chronic GVHD mice (Table II). Moreover, striking elevations in serum IFN-γ levels are seen in acute GVHD which are several logs greater than those of either chronic GVHD mice or control F_1 mice. It should be noted that serum IFN-γ levels in chronic GVHD mice, while significantly less than those of acute GVHD mice, are nevertheless significantly greater than control mice. By day 9 after parental cell transfer, an ~5-fold elevation in serum IL-2 levels is seen for both acute and chronic GVHD mice compared with control F_1 mice ($p < 0.01$, acute GVHD or chronic GVHD vs normal; p = NS, acute vs chronic). Additionally, chronic GVHD mice exhibited an approximate 4-fold elevation in serum IL-4 compared with control F_1 mice ($p < 0.01$); however, acute GVHD mice exhibited even greater elevations in serum IL-4 levels compared with either control F_1 mice (≥9-fold, $p < 0.01$) or chronic GVHD mice (2-fold, $p < 0.01$). These data indicate that although significant increases in cytokine production are present in both forms of GVHD, acute GVHD mice make greater amounts of TNF-α, IL-4, and most notably IFN-γ compared with chronic GVHD mice.

In vivo blockade of TNF-α in acute GVHD selectively blocks production of IFN-γ

Activation of donor $CD4^+$ T cells is a common feature of both acute and chronic GVHD and initially results in the production of IL-2, IL-4, and IL-10 (14). In acute GVHD, activation of donor $CD4^+$ T cells leads to donor $CD8^+$ T cell activation which results in IFN-γ production and the development of an antihost CTL response (14). In contrast, donor $CD8^+$ T cell activation and marked IFN-γ production are not features of chronic GVHD. Because early administration of anti-TNF-α mAb inhibits CTL development (Fig. 2) and IFN-γ contributes to CTL development in this model (14, 18), it was possible that anti-TNF-α mAb blocked CTL development, in part, by inhibiting an IFN-γ response. To determine whether a TNF-α blockade alters cytokine production in acute GVHD, splenic mRNA was assessed for cytokine gene expression by semi-quantitative RT-PCR. As shown in Fig. 3*A*, in vivo treatment with anti-TNF-α mAb resulted in an ~3-fold inhibition of IFN-γ mRNA expression as compared with untreated or control mAb-treated BDF_1 mice that had been inoculated with B6 spleen cells. In contrast, anti-TNF-α mAb treatment did not significantly inhibit IL-4 or IL-10 mRNA expression. These results were confirmed at the level of serum cytokine protein. As shown above in Table II, acute GVHD mice exhibit very high serum levels of IFN-γ at day 6 after parental cell transfer. Anti-TNF-α treatment completely inhibits the acute GVHD-associated rise in serum IFN-γ but does not significantly alter the serum levels of a B cell stimulatory cytokine such as IL-6 (Fig. 3*B*).

TNF-α blockade impairs IFN-γ-mediated Fas up-regulation in acute GVHD

We have previously shown that elimination of host B cells in acute GVHD by donor CTL involves both a Fas/FasL pathway and a perforin pathway (18). Moreover, significant Fas up-regulation on host B cells is characteristic of acute GVHD, but not chronic GVHD, and is largely IFN-γ dependent (18). To determine whether the striking reduction in serum IFN-γ in anti-TNF-α-treated acute GVHD mice results in functional consequences, IFN-γ-dependent Fas expression on host B cells was examined by flow cytometry at 10 days after parental cell transfer. As shown in Fig. 4, *A* and *B*, the characteristic up-regulation of Fas on host B cells in acute GVHD is mostly, but not completely, down-regulated in mice receiving anti-TNF-α mAb. These results are consistent with previous work demonstrating a comparable degree of Fas down-regulation in chronic GVHD mice or in anti-IFN-γ mAb-treated acute GVHD mice (18).

Discussion

Although controversial, human and murine studies suggest a link between reduced TNF-α production and the development of humoral autoimmunity. For example, lupus-prone New Zealand Black/White (NZB/W) mice express an allelic form of the *TNF-α* gene that is associated with decreased TNF-α production (25). Treatment of NZB/W mice with anti-TNF-α mAb exacerbates renal disease and mortality (25), while administration of rTNF-α at

Table II. *Acute GVHD mice exhibit elevations of serum IFN-γ and TNF-α compared to chronic GVHD mice at day 6 after parental cell transfer*

Group[a]	IFN-γ	TNF-α	IL-2	IL-4
Normal F_1	≤40	69 (4)	57 (6)	12 (1)
Acute GVHD	154,476 (7,815)[b,c]	265 (46)[b,c]	344 (72)[b]	104 (14)[b,c]
Chronic GVHD	297 (35)[b]	118 (13)	298 (35)[b]	51 (4)[b]

[a] Acute and chronic GVHD were induced and serum cytokines measured by CCCA as described in *Materials and Methods*. Mice were injected with 10 μg of biotin anti-IFN-γ and anti-TNF-mAb on day 5 and anti-IL-4 and anti-IL-2 mAbs on day 8 and bled on days 6 and 9. Results are shown as mean picograms per milliliter per group with SE in parentheses; n = 4–5 mice/group.

[b] $p < 0.01$ compared to control.

[c] $p < 0.01$ compared to chronic GVHD.

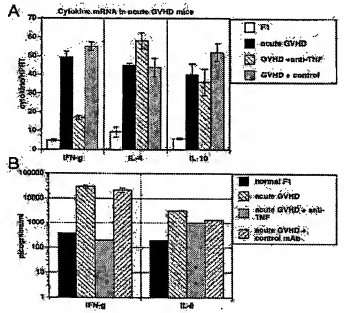

FIGURE 3. In vivo blockade of TNF-α inhibits (*A*) cytokine gene expression for IFN-γ but not for IL-4 or IL-10 and (*B*) serum levels of IFN-γ but not IL-6. Acute GVHD was induced and anti-TNF-α or control mAb was administered as described for Table I. *A*, splenic cytokine gene expression was determined by RT-PCR as described in *Materials and Methods* at 14 days after parental cell transfer. *B*, serum cytokines were determined by CCCA on day 6 for IFN-γ and day 9 for IL-6. Results are shown as the group mean (n = 4–5 mice/group). Similar results have been seen in a second independent experiment.

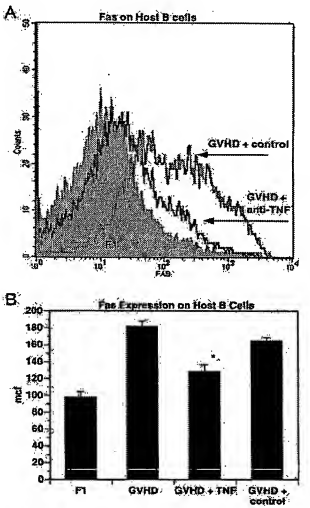

FIGURE 4. Neutralization of TNF-α in vivo blocks up-regulation of Fas on host B cells in acute GVHD. Acute GVHD was induced and anti-TNF mAb or control mAb administered as described for Table I. At day 10 after parental cell transfer, Fas expression was determined on gated host B cells (B220-positive, I-A^d-positive). *A*, Representative tracings are shown for untreated F_1 mice (shaded curve), acute GVHD + control mAb and acute GVHD + anti-TNF-α mAb. *B*, Group mean channel fluorescence values (mean ± SE) are shown, n = 5 mice/group. Similar results were seen in an additional independent experiment. *, $p < 0.01$ compared with untreated GVHD or control mAb treated acute GVHD.

the proper time can retard disease development (26, 27). Furthermore, deletion of the type I *TNFR* gene from C57BL/6.*lpr* mice accelerates the development of lymphadenopathy and autoimmunity (28), whereas breeding a defective *TNF* gene onto New Zealand Black mice enhances the otherwise mild autoimmune response in these mice and results in severe renal diseases similar to NZB/W mice (29). In humans, defective TNF-α responses have been widely reported in association with SLE (10, 30–32) and have been linked to the development of lupus nephritis (10), although not all workers have observed this relationship (33). Lastly, the development of the anti-nuclear Ab, anti-dsDNA Ab and, occasionally, clinical SLE in patients treated with TNF antagonists (4, 7, 34) supports the idea that in some individuals, reduced TNF activity is associated with humoral autoimmunity.

The present study demonstrates that TNF-α is critical for the induction of CTL in vivo and suggests a mechanism by which reduced TNF-α activity may contribute to the development of humoral autoimmunity in both mouse and human. In the parent→F_1 GVHD model, both acute and chronic GVHD are initiated by the activation of donor $CD4^+$ T cells that produce IL-2 and mature into effector T helper cells that activate B cells to proliferate and secrete Ig. As a result, polyclonal B cell activation and autoantibody production can be observed 7–10 days after parental cell transfer in both forms of GVHD (14). Activation of donor $CD8^+$ T cells, which mature into antihost CTL, eliminates autoantibody-secreting host B cells and serves to differentiate acute GVHD from chronic GVHD. Actions that selectively impair $CD8^+$ T cell differentiation into antihost CTL, such as in vivo treatment with anti-IL-2 mAb (15), depletion of donor $CD8^+$ T cells before parental cell transfer (16), or deletion of the perforin gene from donor $CD8^+$ T cells (11), convert acute GVHD to chronic lupus-like GVHD. Thus, CTL likely prevent lupus-like humoral autoimmunity in parent→F_1 GVHD by eliminating autoreactive host B cells. Our results indicate that neutralization of TNF-α during the first few days after parental cell transfer selectively inhibits $CD8^+$ T cell maturation into CTL effectors, in association with suppression of IFN-γ production and decreased Fas/FasL up-regulation. Because anti-TNF-α mAb treatment does not inhibit all cytokine production or B cell hyperactivity, mice then develop chronic lupus-like GVHD.

Although TNF-α is well known to be involved in the CTL effector function, a role for TNF-α in CTL generation has only recently been suggested. T cells from TNFR I-deficient mice exhibit reduced in vitro production of IFN-γ and IL-2 in response to alloantigen (35) and anti-TNF-α Ab has been described to decrease CTL generation, reduce splenomegaly and gastrointestinal pathology, decrease weight loss, and improve survival in an irradiated recipient model of bone marrow transplantation and GVHD and in a parent→F_1 model of GVHD (36–39). In addition, impaired CTL function and reduced Th1 cytokine production were observed in GVHD when donor cells were obtained from TNFR p55-deficient

mice (35). However, previous studies did not investigate the connection between anti-TNF-α treatment and the development of autoimmunity.

Our finding that TNF-α is critical for CTL induction and subsequent control of autoreactive B cells ties together previous unlinked clinical and experimental observations that: 1) TNF-α suppresses humoral autoimmunity (26, 27); 2) TNF-α can induce IFN-γ production (40, 41); 3) IFN-γ enhances CTL function by up-regulating Fas and FasL expression (18); and 4) CTL suppress humoral autoimmunity by killing autoreactive B cells (11, 16). Our studies do not eliminate the possibility that TNF-α may also contribute to CTL activation by up-regulating perforin expression, either through an IFN-γ-dependent or independent mechanism.

Lastly, our results establish a mechanism by which treatment of autoimmune disease patients with TNF-α antagonists could induce or exacerbate disorders of humoral autoimmunity, such as SLE. Although our results by no means argue against the clinical use of TNF-α antagonists which have been remarkably effective therapies for many patients with rheumatoid arthritis or Crohn's disease, they underscore the need to carefully monitor treated patients and to identify factors that might predispose patients to develop autoimmune pathology when treated with TNF-α antagonists. In addition, the possibility that TNF-α may be a general requirement for CTL development should promote caution in using TNF antagonists in patients with conditions in which CTL appear to limit severity, such as viral infections. Conversely, TNF antagonists may be of benefit in conditions in which CTL are detrimental, such as acute allograft rejection.

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